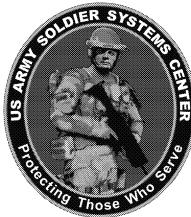


TECHNICAL REPORT
NATICK/TR-07/002



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DEVELOPMENT OF VOLATILE OIL OF MUSTARD AND VANILLIN AS AN EFFECTIVE FOOD PRESERVATION SYSTEM FOR MILITARY BREAD AND BAKED GOODS

by
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Approved for public release; distribution is unlimited

**U.S. Army Research, Development and Engineering Command
Natick Soldier Center
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14. ABSTRACT The objective of the study was to determine if the combination of volatile oil of mustard (VOM) vanillin is an effective food preservation system for molds and yeast. Four bread spoilage organisms were evaluated in the study: Penicillium notatum, Rhizopus stolonifer, Aspergillus niger and Saccharomyces cerevisiae. The VOM/vanillin preservation system was evaluated in an impermeable packaging material developed by the military. Challenge studies were done in both potato dextrose agar (PDA) and military bread. The effectiveness of the preservation system was determined by growth/no growth criteria. The concentration of vanillin or VOM needed to inhibit each spoilage organism varied. Aspergillus niger was the most resistant organism to vanillin, > 2000 ppm was needed for inhibition. In contrast, Rhizopus stolonifer was resistant to VOM. However, when the combination of VOM/vanillin was used together there was an additive effect or possibly a synergistic effect on both organisms. It was determined that 1500 ppm of vanillin in bread and 155 ug/L of VOM in the headspace of the packaging inhibited the outgrowth of both organisms for greater than three months. These results suggest that the preservation system of VOM/vanillin can effectively replace the oxygen scavenger.											
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Table of Contents

List of Figures and Tables.....	iv
Preface.....	v
Summary.....	1
Introduction.....	2
Materials & Methods.....	2
Model System.....	2
VOM/Vanillin in Combination (Model System).....	3
VOM/Vanillin in Combination (Bread Product).....	3
Results.....	4
Discussion.....	10
Conclusions.....	11
References.....	13

List of Figures

Figure	Page
1. Shows the growth of <i>Aspergillus niger</i> in the model system at different concentrations of vanillin.	5
2. Shows the growth of <i>Aspergillus niger</i> in the model system in the presence of different concentrations of VOM.	6
3. Observation of bread samples immediately after being removed from MRE package. Two breads on left were control samples and bread on far right is treated bread sample with VOM/vanillin.	9
4. Observation of bread samples after challenge study in which the breads were left on lab bench for two weeks after test. Bread on left is untreated bread and bread sample on right had been treated with VOM/vanillin.	10

List of Tables

Table	Page
1. The table shows MRE bread recipe with ingredients weight in grams. Vanillin content of bread varied in percentages of 0.15 % and 0.2 %.	4
2. Summary of inhibitory concentrations of vanillin and VOM (AIT) necessary to prevent the growth of <i>Saccharomyces fibuligera</i> , <i>Rhizopus stolonifer</i> , <i>Aspergillus niger</i> and <i>Penicillium notatum</i> in the model system.	5
3. The growth or no growth of <i>Aspergillus niger</i> in the model system in the presence of different concentrations of VOM (AIT active agent) and vanillin.	7
4. The growth or no growth of <i>Rhizopus stonoifer</i> in the model system in the presence of different concentrations of VOM (AIT active agent) and vanillin.	7
5. The growth or no growth of <i>Aspergillus niger</i> and <i>Rhizopus stolonifer</i> in the model system in the presence of different concentrations of VOM (AIT, active agent) and vanillin.	8
6. The results of bread challenge study in which <i>A. niger</i> and <i>R. stolonifer</i> were inoculated into bread samples. The packaged bread samples were treated with VOM/vanillin in combination.	9

Preface

This report summarizes the in-house work on the project performed under the Biosystems Technology Program (BTP), program element number PE 63001. The BTP, a congressionally-funded program, focused on environmentally preferable and responsible products and services derived from tropical plants and microorganisms, and restoration of contaminated resources. The BTP was also intended to support sustainable regional economics development through technology transfer and commercialization of products and services of interest to military and civilian markets. The program addressed needs and opportunities in Hawaii and the Asia-Pacific region.

This project was entitled “Hawaiian Vanilla as a Military/Commercial Product.” The project was funded from June 2002 to June 2003. One part of the project was to compare Hawaiian vanilla with other commercially available vanillas produced throughout the world. The commercial vanilla samples originated from various geographical locations, including Mexico, Indonesia, Madagascar, Tahiti and Uganda. The Hawaiian vanilla was compared with commercial samples for its aromatic attributes to determine its commercial viability. This part of the study was contracted out to Sensory Spectrum, Inc. and is not covered in this Technical Report. This report covers the in-house work of developing a food preservative from natural products volatile oil of mustard (VOM) and vanillin.

Summary

Congressional funds were appropriated to revitalize the economy in Hawaii by developing products unique to the state. This project looked at vanilla and related extracts. The Hawaiian Vanilla Company is the only commercially operated vanilla company in the United States. There was a need to establish the quality of vanilla produced in Hawaii. To do this, a scientifically-based sensory analysis was needed to compare Hawaiian vanilla with other major commercial vanilla producers. This information was essential to publicize and establish Hawaiian vanilla in the world commercial market. That portion of the project was handled by a contractor, Sensory Spectrum, Inc. This report covers the second part of the project the functional properties of vanilla and other related extracts.

Vanilla is an aromatic compound that can be used as a masking, antimicrobial and antioxidant agent. It has been shown in the literature that vanilla and vanillin, a major component of vanilla, demonstrate these properties in various products. At the US Army Natick Army Soldier Center, researchers evaluated the antimicrobial properties of vanilla, vanillin and volatile oil of mustard (VOM) (active component, allyl isothiocyanate). It was determined that vanilla was not a good antimicrobial and its cost would be cost prohibited in military products. However, the antimicrobial inherent qualities of vanillin and VOM were very intriguing. Both vanillin and VOM exhibit inhibitory activity against bacteria, fungi, and molds. Both of these compounds are volatile, with VOM having a very pungent odor versus vanillin's rather pleasant odor. It was determined in this study that when used in combination VOM and vanilla can be a very effective food preservative for breads and other baked goods.

DEVELOPMENT OF VOLATILE OIL OF MUSATARD AND VANILLIN AS AN EFFECTIVE FOOD PRESERVATION SYSTEM FOR MILITARY BREAD AND BAKED GOODS

Introduction

In the development of military food it is necessary to take into consideration unique requirements that are outside the commercial sector. For instance, MRE military bread requires a storage period of three years and must withstand extreme environmental conditions. Due to these requirements the bread is specially formulated to prevent spoilage and sealed in an impermeable package material with an oxygen scavenger. Even with these demanding requirements there has been an increased emphasis, in the military as well as the commercial sector, on natural preservatives. In addition, the military would like to eliminate the use of oxygen scavenger from their products.

In this study two natural antimicrobial compounds are evaluated in a model system and in a challenge study in a military product. The two antimicrobial compounds are vanillin and volatile oil of mustard (VOM) or WasaOuroTM (active ingredient allyl isothiocyanate, AIT). VOM, whose active ingredient, AIT, is derived from mustard seed extract, has a very pungent smell resembling that of horseradish. Vanillin on the other hand has a very pleasant smell, very much like vanilla. The combination has the potential to mask any unpleasant odors as well as prove to be an excellent antimicrobial combination to inhibit food spoilage organisms particularly molds. It is believed that the combination may be practical for baked goods such as bread to eliminate the need for an oxygen scavenger in the Meals Ready to Eat (MRE) package.

In the model system the antimicrobial properties of VOM and vanillin were evaluated alone against four organisms associated with bread. The organisms selected were *Penicillium notatum*, *Rhizopus stolonifer*, *Aspergillus niger*, and *Saccharomyces cerevisiae*. Then the two antimicrobial agents were evaluated together against two of the most resistant organisms tested, to determine if the combination exhibited any synergistic properties. After determining the relative effective concentration of vanillin/VOM in the model system, a challenge study was designed to determine the antimicrobial effectiveness of the combination in MRE bread. The vanilla/VOM combination appears to be effective against *Aspergillus niger* and *Rhizopus stolonifer* in both the model system and the MRE bread product.

Materials & Methods

I. Model System

The model system consisted of a Petri dish (60 mm x 15 mm) containing a Gelman cellulose filter pad to hold a prepared measured quantity of VOM

(WasaOuroTM) solution. Another Petri dish (100mm x 15mm) having potato dextrose agar (PDA agar), with and without vanillin, was inoculated with the appropriate organism, inoculum 105 spores/ml. The Petri dishes are uncovered, placed in a MRE impermeable laminated package and the package heat-sealed. The MRE packages are then incubated at 25° C for seven days.

Four organisms were studied: *Penicillium notatum*, *Rhizopus stolonifer*, *Aspergillus niger* and *Saccharomyces fibuligera*. The determination of effectiveness of the antimicrobial agents was based on a growth or no growth bases. The plates with no growth were left out for seven days after removal from the MRE package to ensure there was no additional growth.

Vanillin was placed in the PDA agar at 250 ppm increments, 250 – 2000 ppm. A stock solution of the VOM (100mg/ml) was made and 100, 200, 400, 600, 800, and 1000 ul were added to the filter pads. The amount of the volatile active component of VOM, AIT, was determined using gas chromatography. For each experiment a set of control MRE packages were made with the various concentrations of VOM. These packages contained no organism or agar. The determined AIT amounts in the control packages were used to extrapolate the relative AIT in the experimental packages.

II. VOM/Vanillin in Combination (Model System)

The same model system was used to determine the antimicrobial effectiveness of the combination of VOM/vanillin. There were two changes in the model system. Instead of using a soluble powder for delivery of VOM, an adhesive polymer label was used containing a specific amount of VOM. These labels are available from the same manufacturer of the VOM powder. The labels are easier to use than the powder. As before, the VOM labels were evaluated using gas chromatography to measure the amount of volatile AIT in the sealed MRE package.

The second change was only two of the most resistant organism to VOM and vanillin were tested as determined from the previous experiments. The two organisms tested were *Aspergillus niger* (most resistant organism tested against vanillin) and *Rhizopus stolonifer* (resistant to VOM). The two organisms were tested against VOM/vanillin alone and together in the model system. As before, the samples were incubated at 25° C for seven days and rated on a growth/no growth criteria.

III. VOM/Vanillin in Combination (Bread Product)

A challenge study was designed in which the VOM/vanillin combination was used in MRE bread and package material. The vanillin was added to the bread recipe at appropriate concentrations and VOM labels attached and enclosed in the MRE package containing the bread. The oxygen scavengers usually used

in the MRE packages were omitted. All breads in challenge study were inoculated with spores of both *Aspergillus niger* and *Rhizopus stolonifer*.

Two different concentrations of vanillin were incorporated in the bread 0.15% (1500 ppm) and 0.20% (2000 ppm). In each package containing VOM, a half of VOM label (40 mm X 40 mm) was attached to the inside of the MRE package to deliver the required concentration of AIT. This would produce at time 0 approximately 155 ug/ml of AIT in the MRE package. Two control samples were made, one having VOM and another no VOM. The two control sample breads had no vanillin. The samples were incubated at 25° C and analyzed at 0, 7, 21, 35, 49, 63, 77, 91, and 105 days for growth. At each sampling time there were three samples for the controls and VOM/vanillin concentrations.

MRE Bread Recipe Containing Vanillin

Table 1. The table shows MRE bread recipe with ingredients weight in grams. Vanillin content of bread varied in percentages of 0.15 % and 0.2 %.

Ingredients	Percent	Control	0.15 %Vanillin	0.2 % Vanillin
Bread Flour	50.25	1206	1206	1206
Yeast	0.9	21.6	21.6	21.6
Salt	1.29	30.96	30.96	30.96
Sucrose Ester	1	24	24	24
Xantham Gum	0.5	12	12	12
Gum Arabic	0.25	6	6	6
Calcium	0.25	6	6	6
Gluten	0.98	23.52	23.52	23.52
Vanillin USP	varied	0	3.6	4.8
Water	28.66	687.84	687.84	687.84
Glycerol	6.36	152.64	152.64	152.64
Shortening	8.58	204.72	204.72	204.72

Results

Four different yeast and mold organisms associated with the spoilage of bread were tested in the model system. The organisms were *Saccharomyces fibuligera*, *Rhizopus stolonifer*, *Aspergillus niger* and *Penicillium notatum*. Each organism was tested separately against VOM and vanillin to determine their relative sensitivity to each of these compounds. The table (Table 2) that follows shows the summary of results obtained against each organism indicating the relative range of concentration required to prevent growth of each organism in model system. Also Fig. 1 and Fig. 2 show how the samples were treated and the results for one particular organism, *Aspergillus niger*, for vanillin and VOM. An asterisk was used to designate the most resistant organism to each of the two compounds tested.

Table 2. Summary of inhibitory concentrations of vanillin and VOM (AIT) necessary to prevent the growth of *Saccharomyces fibuligera*, *Rhizopus stolonifer*, *Aspergillus niger* and *Penicillium notatum* in the model system.

<u>Organism</u>	<u>Vanillin</u>
<i>Saccharomyces fibuligera</i>	>1000ppm - <1250ppm
<i>Rhizopus stolonifer</i>	>1000ppm - <1250ppm
<i>Aspergillus niger</i> *	>1750ppm - <2000ppm
<i>Penicillium notatum</i>	> 500ppm - < 750ppm

<u>Organism</u>	<u>VOM (AIT)</u>
<i>Saccharomyces fibuligera</i>	~ 8 ppm
<i>Aspergillus niger</i>	~17 ppm
<i>Penicillium notatum</i>	~17 ppm
<i>Rhizopus stolonifer</i> *	> 470 ppm (resistant)

* Indicates the most resistant organism to each agent

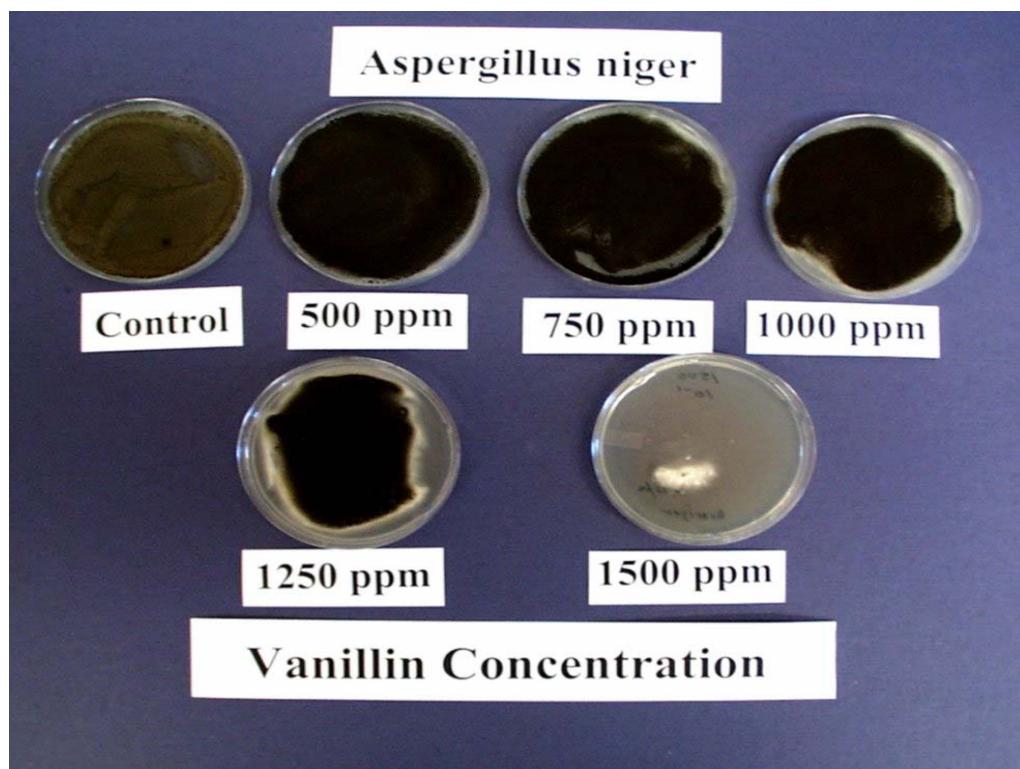


Figure 1. Shows the growth of *Aspergillus niger* in the model system at different concentrations of vanillin.

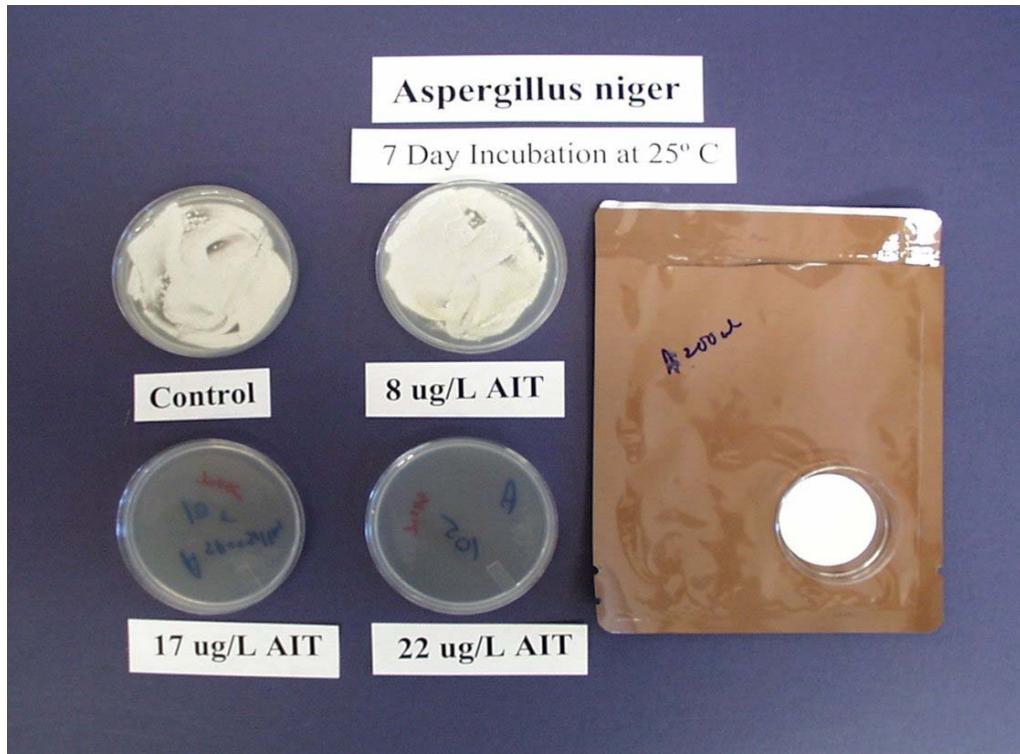


Figure 2. Shows the growth of *Aspergillus niger* in the model system in the presence of different concentrations of VOM.

It was determined that *Aspergillus niger* was the most resistant against the effect of vanillin while *Rhizopus stonoifer* was highly resistant to VOM. Once the two most resistant organisms to the two compounds were determined the model system was used to test both VOM and vanillin together against each of these organisms. The only change in the model system besides using the compounds together was how the VOM was to be delivered. Instead of a soluble powder an adhesive label, containing a designated amount of VOM, (AIT) was used inside the impermeable laminated MRE packages.

Table 3 shows the growth of *Aspergillus niger* in the presence of different concentrations of VOM and vanillin. The squares marked “delayed”, indicates the growth of the organism after they were removed from MRE package and Petri plate left on the lab bench at room temperature. The “NA” designation indicates no data available. No samples were made for these concentrations. Table 4 represents the growth of *Rhizopus stonoifer* in the presence of different concentrations of VOM and vanillin. Finally table 5 represents the growth or no growth of both organism in the presence of different concentrations of VOM and vanillin. The designation of “sensory neutral” indicates the odor of package immediately after being opened. The observation was made to see if at these higher levels of VOM and no growth that the sensory aspects of the product were tolerable. There were no noticeable offensive odors at these levels.

Table 3. The growth or no growth of *Aspergillus niger* in the model system in the presence of different concentrations of VOM (AIT active agent) and vanillin.

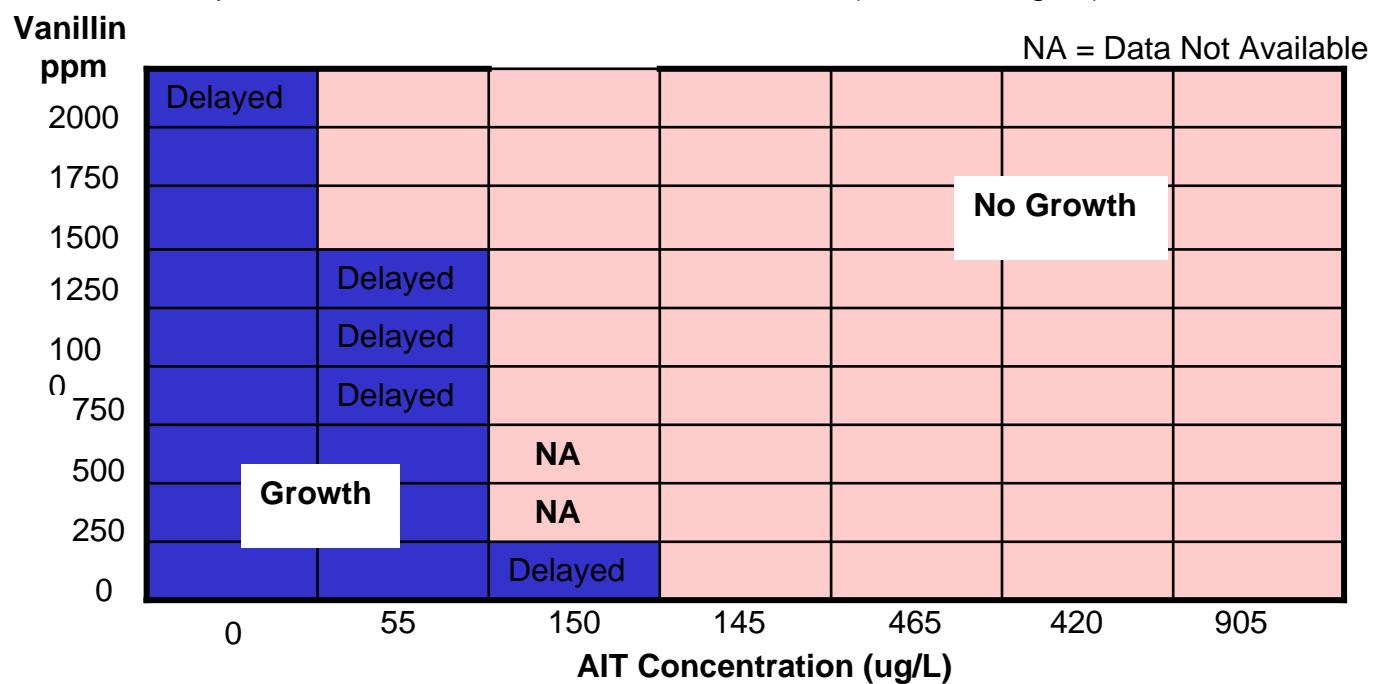


Table 4. The growth or no growth of *Rhizopus stonoifer* in the model system in the presence of different concentrations of VOM (AIT active agent) and vanillin.

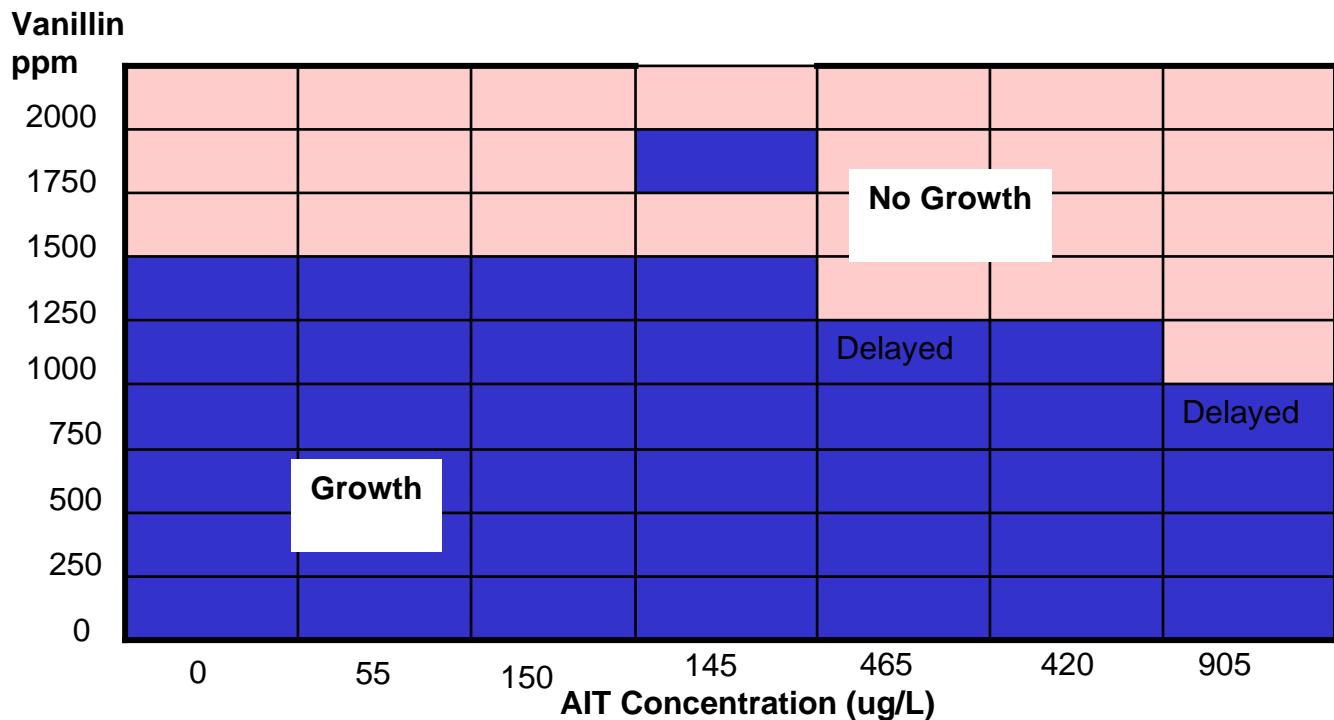
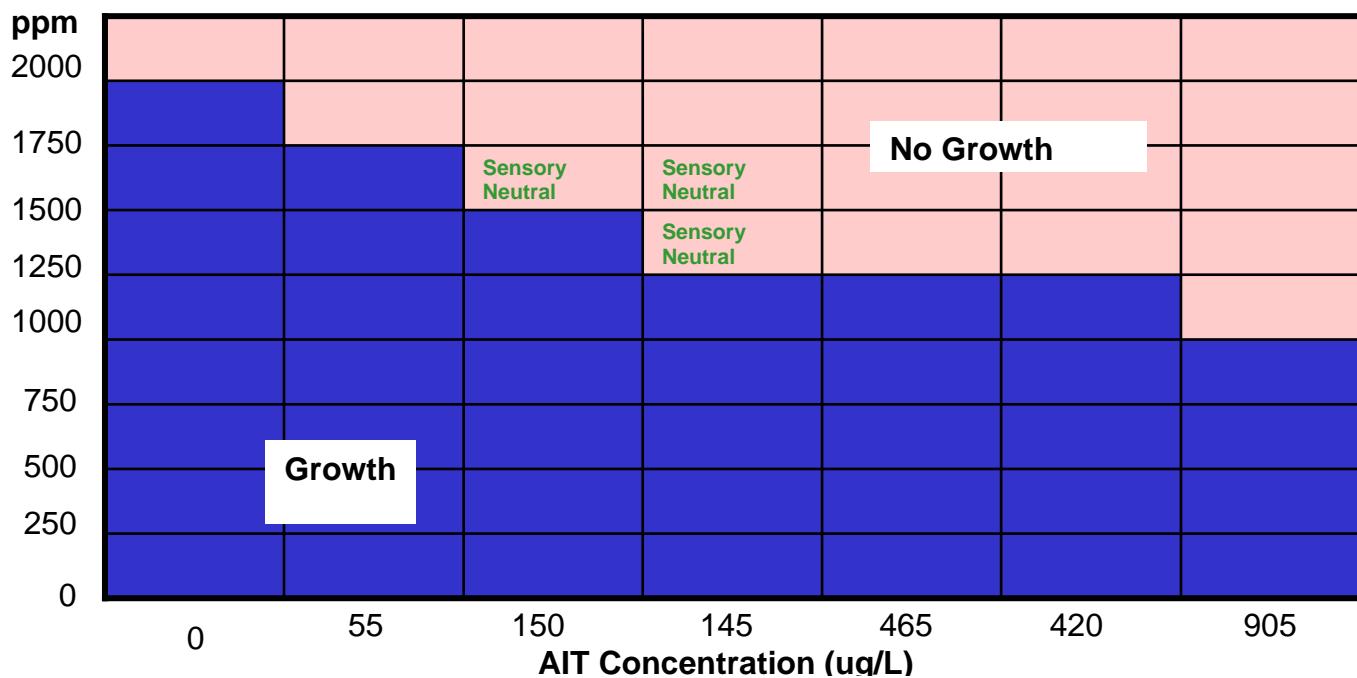


Table 5. The growth or no growth of *Aspergillus niger* and *Rhizopus stolonifer* in the model system in the presence of different concentrations of VOM (AIT, active agent) and vanillin.

Vanillin



After determining the appropriate concentrations of VOM and vanilla that would inhibit the growth of *A. niger* and *R. stolonifer* in the model system a challenge study was designed using MRE bread. Two different concentrations of vanillin were incorporated in the bread 0.15% (1500 ppm) and 0.20 % (2000 ppm). In each package an adhesive label was incorporated in the package to deliver approximately 155 ug/ml of AIT. The oxygen scavengers usually used in the MRE packages were omitted as well as potassium sorbate from the bread recipe.

Two control samples were made, one having VOM and another no VOM. The two control sample breads had no vanillin. The samples were incubated at 25° C for 105 days with a number sampling times. Table 6 shows the results of the challenge study. The samples were observed at each sampling time for any indication of growth of the two organisms, *A. niger* and *R. stolonifer*. Over the 105 days no growth was shown by bread treated with VOM/vanillin. Fig. 3 shows the samples immediately after packages were open. The two control samples exhibited growth on the bread while VOM/vanillin treated sample showed no growth. A treated and untreated bread samples were removed from packaging and left out on lab bench for two weeks. Fig. 4 shows the results of leaving the treated and untreated bread samples on lab bench at room temperature. The untreated bread was overwhelmed with growth while the treated bread sample remained unaffected.

Table 6. The results of bread challenge study in which *A. niger* and *R. stolonifer* were inoculated into bread samples. The packaged bread samples were treated with VOM/vanillin in combination.

Time (Days)	Control (no VOM)	Control (VOM)	0.15% Vanillin + VOM	0.2 % Vanillin + VOM
0	0/3	0/3	0/3	0/3
7	3/3	0/3	0/3	0/3
21	3/3	0/3	0/3	0/3
35	3/3	1/3	0/3	0/3
49	3/3	1/3	0/3	0/3
63	3/3	1/3	0/3	0/3
77	3/3	1/3	0/3	0/3
91	3/3	1/3	0/3	0/3
105	3/3	1/3	0/3	0/3

Sampling = Visible Growth/# of Samples



Figure 3. Observation of bread samples immediately after being removed from MRE package. Two breads on left were control samples and bread on far right is treated bread sample with VOM/vanillin.



Figure 4. Observation of bread samples after challenge study in which the breads were left on lab bench for two weeks after test. Bread on left is untreated bread and bread sample on right had been treated with VOM/vanillin.

Discussion

The antimicrobial effectiveness of VOM and vanillin against yeast and molds in the model system are shown in Table 2. The organisms tested were *Saccharomyces fibuligera*, *Rhizopus stolonifer*, *Aspergillus niger* and *Penicillium notatum*. *Saccharomyces* is the most sensitive organism to VOM and *Rhizopus* appears to be resistant to VOM (active ingredient AIT), at least resistant to the highest levels used in this study. Vanillin was most effective against *Penicillium* while *Aspergillus* showed the most resistance to vanillin of the organism tested. Figs. 1 and 2 show the results obtained against *Aspergillus*. All organisms tested are associated with the bread spoilage, the product of interest in this study.

Vanilla, to be an effective antimicrobial has to be in contact with organism. Vanillin is a food-grade ingredient and for this reason vanillin can be incorporated into the product of interest. Also it has a pleasant aroma, similar to vanilla. On the other hand VOM, the active ingredient being AIT, is a volatile compound. For this reason it is incorporated in the packaging material of the model system. It is not necessary to be in direct contact with the product to be effective. The VOM, however, has a strong pungent smell that can be overpowering. That is why the combination of VOM/vanilla maybe a very interesting combination as a preservative for the appropriate products such as baked goods. The vanilla will mask the unpleasant odor of the VOM and the combination will provide the necessary antimicrobial protection of product.

In Tables 3, 4, & 5 the results indicate that VOM/vanillin can be an effective preservation combination against mold and yeast. In addition the combination may have a synergistic effect in inhibiting the two most resistant organisms tested, *Aspergillus* and *Rhizopus*. Table 3 indicates how sensitive *Aspergillus* is to VOM showing no growth at the second lowest level of VOM used in the study. *Rhizopus* on the other hand in Table 4 shows how resistant it is to VOM. However, it appears it is vulnerable when vanillin is added to the equation. It appears to be an additive effect if not a synergistic effect, as the concentration of VOM is increased the amount of vanillin necessary to inhibit the outgrowth of the spores decreases. In Table 5 both *Aspergillus* and *Rhizopus* are being tested against VOM/vanilla. The table appears to show that inhibition of the two organisms and Table 5 is a composite of Tables 3 and 4, as would be expected.

Based on the data from the model system an effective concentration range for the VOM and vanillin was determined so a challenge study could be designed for the MRE bread. Both the oxygen scavenger and a preservative ingredient usually incorporated in the bread recipe, potassium sorbate, were omitted to determine effectiveness of the combination. Two vanillin concentration were used, 0.15% and 0.20%, and an AIT concentration of approximately 155 ug/L. Table 6 shows the results. The bread was inoculated with *Aspergillus* and *Rhizopus* spores in different areas on the bread surface. The concentrations of vanillin and the VOM concentration effectively prevented the outgrowth of both organisms. In photo 3, the two controls, without VOM/vanillin treatment, show the evidence of growth of the two organisms. The third bread in the photo is treated with VOM/vanillin, at the same sampling time, having no growth. Fig. 4 is the observation of two breads after the challenge study, one untreated and the other bread treated with VOM/vanilla, after being left out on lab bench at room temperature for two weeks. The untreated (control) bread was covered with mold, the treated bread showed no evidence of mold growth.

Conclusions

Aspergillus niger, *Rhizopus stolonifer*, *Penicillium notatum*, and *Saccharomyces fibulgera* are sensitive to vanillin.

Three of the organisms are sensitive to VOM with *Rhizopus stolonifer* exhibiting a high resistance to AIT, the active component of VOM.

The combination of VOM/vanilla was effective in inhibiting both *Aspergillus* and *Rhizopus*, the most resistant organisms tested.

VOM/vanilla combination exhibits an additive effect if not a synergistic antimicrobial effect against *Rhizopus* in model system.

Challenge study in bread indicates VOM/vanilla was an effective preservation system protecting the bread for over three months.

By using the combination of VOM/vanillin it may be possible to eliminate the need for an oxygen scavenger and potassium sorbate in the MRE bread.

The VOM/vanillin combination may have a number of possible applications as a preservative in military and commercial products.

This document reports research undertaken at the U.S. Army Research, Development and Engineering Command, Natick Soldier Center, Natick, MA., and has been assigned No. Natick/TR-07/002 in a series of reports approved for publication.

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